Biochemical and Cytochemical Comparison of Surface Membranes from Normal and Dystrophic Chickens

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Cytochemical and biochemical characteristics of the surface membrane components of avian dystrophic muscle were examined. A Mg^{2+}- or Ca^{2+}-activated ("basic") adenosine triphosphate (ATPase) was localized cytochemically in fixed, intact dystrophic muscle slices in a medium containing Mg^{2+} or Ca^{2+}, adenosine triphosphate (ATP), and 1 μ M free Pb^{2+} to capture enzymatically released phosphate ions. Electron-dense staining precipitates were found to be associated with the plasmalemma and its tortuous invaginations, and the transverse components of the T-system membrane and its associated proliferated networks. Enzymatic analysis of microsomal fractions isolated from 7-day-old and 90-day-old normal and dystrophic muscle showed a complex behavior. Specific activity of "basic" ATPase decreased with maturity in normal and dystrophic animals. The specific activities of the surface membrane associated enzymes, leucyl β-naphthylamidase, adenylate cyclase, and guanylate cyclase, remained at various elevated levels in the mature dystrophic animals, in contrast to the normal muscle, which showed decreases in the specific activity of all three enzymes with maturation. The persistent high levels in some but not all enzyme activities in 90-day-old dystrophic muscle indicates a complicated developmental pattern in the dystrophic chicken muscle. (Am J Pathol 1981, 105:223–231)

THE SURFACE MEMBRANE components, the cell membrane (plasmalemma) and the transverse tubular system (T system), of pectoral muscle in the dystrophic chicken show proliferative changes reminiscent of normal embryonic avian pectoral muscle grown uninnervated in *in vitro* cultures[1] or of human muscle grown subcutaneously in nude mouse.[2] A honeycomb tubular network continuous with vesicular dilatations and scalloped invaginations associated with the T system[3,4] and an increase in the density of invaginated caveolae, together with a topographic disarray of the plasmalemma, are distinct features of the dystrophic muscle.[5]

Recently we have localized a "basic" (Mg^{2+} or Ca^{2+}) adenosine triphosphatase (ATPase) activity associated with striated muscle surface membranes and found the activity to be a good marker for such membranes in normal chicken,[6] rabbit skeletal muscle (unpublished data), and canine myocardium.[7] In the present study, we show that the "basic" ATPase activity, when examined by ultrastructural cytochemical analysis in dystrophic chicken pectoral muscle, exhibits a staining pattern that corresponds to the underlying intact vesicular, undulated, proliferated components of the surface membrane. In addition, we have examined biochemically the changes related to dystrophy in the activity of "basic" ATPase as well as several other enzymes thought to be associated with the skeletal muscle surface membrane. These activities were studied in normal and dystrophic chicken 7 and 90 days old. The sarcoplasmic reticulum Ca^{2+}-ATPase activity was examined for comparison.

Materials and Methods

Dystrophic roosters and hens (line 413) and controls (line 412) were obtained from the Department of Avian Sciences of the University of California at Davis. Animals 7 days old were sacrificed by decapi-

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tation. Animals 90 days old were killed with an overdose of phenobarbitol administered intravenously through an axillary vein.

Cytochemical Methods

"Basic" ATPase activity was localized in thin slices of fixed pectoral muscle as previously described[6] with a modification of the lead precipitation technique of Wachstein and Meisel[8] (reviewed by Firth[9,10]). Unless otherwise indicated, pieces of fresh clamped muscle were placed at 4 C in 5 ml of Buffer A (0.25 M sucrose, 100 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), pH 7.2) containing freshly dissolved 4% paraformaldehyde as fixative and 2 mM ouabain to inhibit (Na^+-K^+)-ATPase. After 30 minutes at 4 C, the muscle pieces were rinsed for 5–10 minutes in two 5-ml washes of buffer A (at 23 C), placed in 0.2 ml of buffer A containing liquified 2.5% agarose, cooled at 0 C, and cut into thin (75 μ) sections with a Sorvall TC2 tissue sectioner (Smith and Farquhar). The thin sections were assayed at 25 C for 20–30 minutes in buffer A to which was added about 3 mM Tirone, 2 mM Pb^{2+}, 5 mM Mg^{2+} or 4 mM Ca^{2+}, and 2 mM ATP. The final free lead concentration was adjusted to about 1 μM with the use of Tirone (catechol-3,5-disulfonic acid)[11,12] by means of a lead electrode. Free lead concentrations of higher that 5 μM resulted in high levels of nonspecific, random "background" staining.

Incubation was terminated by a rinse of the muscle slices in buffer A at 4 C and postfixation at 4 C for 1 hour in 2 ml of buffer A containing 2% glutaraldehyde. The slices were rinsed in 2 ml of 0.25 M sucrose in 0.1 M cacodylate, pH 7.1 (at 4 C), then fixed for 1 hour in 0.1 M cacodylate, pH 7.1 (at 23 C) containing 1% osmium tetroxide (OsO_4), dehydrated in sequential changes of 35%, 70%, 90%, and absolute ethanol, and slowly infiltrated with Epon 812. Small bundles of surface fibers were selected and sectioned on a Porter-Blum Sorvall microtome (MTI) with an MJO diamond knife. The sections were collected on copper grids, stained with 5% aqueous uranyl acetate for 20 minutes and basic lead citrate for 10 minutes, and then examined on a Zeiss model 10A at a voltage of 60 kV. Some sections were examined with no staining.

Cytochemical control experiments to determine the specificity of lead phosphate precipitates were carried out on normal pectoral muscle in conjunction with the dystrophic muscle. These are prescribed in detail in a previous publication.[6] In summary, "basic" ATPase was inactivated by heat (60 C for 40 minutes) after tissue was fixed in 4% paraformaldehyde solution. Preferential lead binding to surface membranes was tested by exposure of fixed muscle slices to lead-Tirone solution that contained no ATP. Subsequently, slices were soaked in Solution A in the presence of 0.1 M phosphate.[11] Interference by the (Na^+-K^+)-ATPase was eliminated with the use of ouabain or a solution containing low K^+ or low Na^+. Another membranous ATPase, that was possibly interfering, the sarcoplasmic reticulum Ca^{2+}-ATPase, was inhibited with aldehyde fixation.[6] Our previous studies showed that aldehyde fixation almost completely inactivated sarcoplasmic reticulum Ca^{2+}-ATPase, whereas the activity of "basic" ATPase was minimally inhibited with 4% paraformaldehyde fixation at 4 C.

Cytochemical localization of sarcoplasmic reticulum Ca^{2+}-ATPase activity was carried out in unfixed tissue, as previously described.[6]

Isolation of Membranes

Membranous fractions derived from surface membranes (plasmalemma and T system) and sarcoplasmic reticulum of normal and dystrophic chicken pectoralis muscle were prepared as previously described[6] with the following modifications. Muscle (5–30 g) was cut into small pieces and homogenized in 20 volumes of 0.3 M sucrose, 20 mM HEPES, pH 7.5, at 0 C for 60 seconds in a Waring Blender. The homogenate was centrifuged for 12 minutes at 8000*g*. A cytosol fraction and a microsomal fraction were obtained from the supernatant by centrifugation for 90 minutes at 100,000*g*. The pellet was resuspended in 0.3 M sucrose and 2.5 mM HEPES, pH 7.4. For further purification, the sample was placed on a sucrose gradient consisting of 10 ml of 25% (w/w), 10 ml of 30%, 8 ml of 34%, and 4 ml of 42% sucrose. Sucrose gradient solutions contained 50 mM KCl and 2.5 mM HEPES, pH 7.4. After centrifugation for 15 hours at 75,000*g*, membranes present at 0.3 M/25% (Fraction 1), 25%/30% (Fraction 2), 30%/34% (Fraction 3), and 34%/42% (Fraction 4) sucrose interfaces were collected, diluted with 1.5 volumes of H_2O, and centrifuged for 90 minutes at 100,000*g*. The pellets were resuspended in a small volume of 0.3 M sucrose, 2.5 mM HEPES, pH 7.4, quick-frozen, and stored at −65 C.

Biochemical Assays

Protein was determined by the procedure of Lowry et al[13] with bovine serum albumin as a standard. Free Pb^{2+} concentrations of histochemical assay mediums were determined with an Orion Lead Electrode (Model 94–82). A standard curve was prepared by addition of increasing amounts of Pb $(NO_3)_2$ to a buffer containing 0.25 M sucrose, 100 mM KCl, and 10 mM HEPES, pH 7.2.

"Basic" ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase activities were determined at 32 C. "Basic" ATPase activity was measured in 2 ml of a medium containing 10 mM HEPES, pH 7.3, 0.1 M KCl, 2.5 mM ATP, 6 mM Mg^{2+} plus 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-N, N'-tetraacetic acid (EGTA), 1 μ mM VO_4^-, and 2.5 μg oligomycin. Ca^{2+}-ATPase was calculated by subtracting "basic" ATPase activity from the ATPase activity measured in 10 mM HEPES, pH 7.3, 0.1 M KCl, 2.5 mM ATP, 5 mM Mg^{2+}, 20 μM Ca^{2+}, 1 μM VO_4^-, 2.5 μg oligomycin, and the ionophore A23187 (2 μg/ml). Vanadate and oligomycin were added to the assay media to inhibit (Na^+-K^+)-ATPase[14] and mitochondrial ATPase activities, respectively. The reactions were started by the addition of ATP and stopped after 2.5, 5, and 10 minutes with 0.7 ml of 1.5 M $HC10_4$. Inorganic phosphate was determined on 1 ml of the protein-free supernatant[15] with Elon as a reducing agent. The enzyme concentrations used resulted in less than 10% hydrolysis of ATP.

The concentration of the Ca^{2+}-dependent and ^{32}P-labeled phosphoenzyme intermediate of sarcoplasmic reticulum was determined under steady state conditions as previously described.[15] Leucyl β-naphthylamidase was determined at 37 C by the method of Goldbarg and Rutenberg,[16] and 5'-nucleotidase activity was measured at 32 C by the method of Michell and Hawthorne.[17] Adenylate cyclase activity was measured in a medium containing 50 mM Tris-HCl, pH 7.7, 10 mM theophylline, 10 mM $MgCl_2$, 10 mM NaF, 20 mM phosphocreatine, 10 μg/ml phosphocreatine kinase (140 units/mg), and 1 mM adenosine triphosphate (ATP). Guanylate cyclase activity was determined in a medium containing 50 mM Tris-HCl, pH 7.7, 0.1 mM isobutylmethylxantine, 5 mM Mn^{2+}, 20 mM phosphocreatine, 10 μg/ml phosphocreatine kinase, and 1 mM guanosine triphosphate (GTP).[18] Samples were preincubated for 10 minutes at 32 C in the absence of the respective triphosphate nucleotide. The reactions were initiated by the addition of 1 mM ATP or GTP, and terminated after 5, 10, and 15 minutes by the addition of 0.6% acetic acid followed by heating at 90 C for 3 minutes. The cyclic AMP and GMP generated in the reactions were measured by the method of Steiner et al[19] with the aid of the New England Nuclear cyclic AMP and GMP radioimmunoassay kits.

Results

Cytochemical Results

Fixed tissue incubated in the presence of ATP (1 mM) and Pb^{2+} (0.5–1 μM free lead) exhibited electron-dense lead phosphate staining associated with surface membranes. The staining precipitate was present over the cytoplasmic side of the plasmalemma and over its extensive caveolar and tubular invaginations (Figure 1). It was associated with the transverse tubules at the triads and was localized on the sarcoplasmic side of the T-system membrane. Staining was also associated with the membranous walls of the honeycomb network aggregates, cisternal dilatations, and invaginations associated with the T system (Figure 2). These structures have been shown by freeze fracture and extracellular tracers to be continuous with the T system.[3] Some electron-dense deposits were present over mitochondria and nuclei. A scant precipitate was seen in the background over

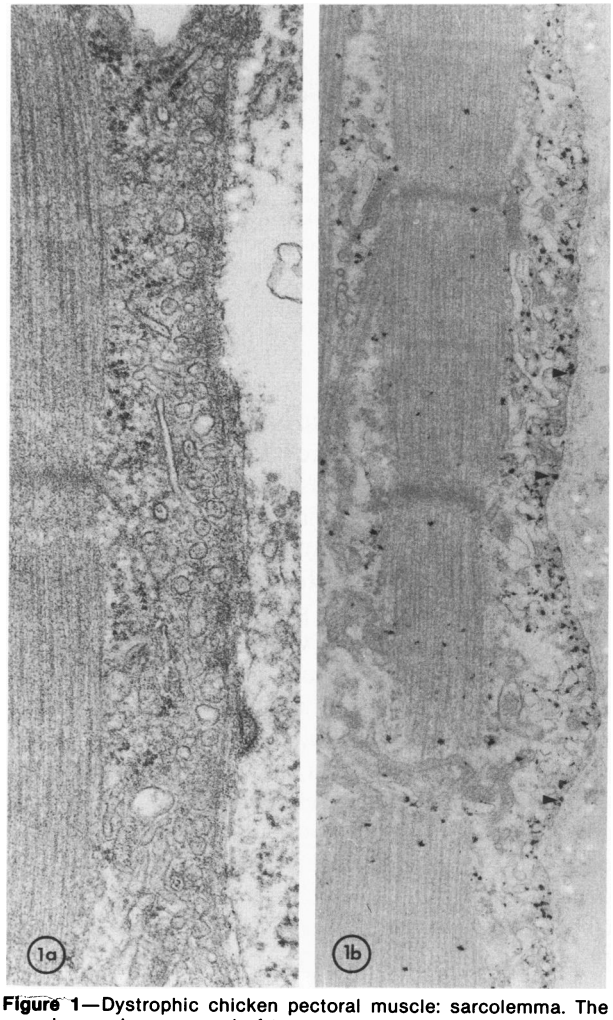

Figure 1—Dystrophic chicken pectoral muscle: sarcolemma. The sarcolemma is composed of two components, an outer basement membrane and an inner plasmalemma, which shows extensive membranous caveolar invaginations in dystrophic muscle (**a**, unstained section). Fixed muscle slices were incubated with buffer A to which was added 2 mM ATP, 5mM Mg^{2+} or 4mM Ca^{2+}, 3mM Tirone, and 2 mM Pb^{2+} to give a free lead concentration of 1 μM (**b**). The plasmalemma exhibits electron-dense staining precipitate associated with the protoplasmic side of the membrane (*arrowheads*) as well as the underlying extensive membranous caveolar and tubular structures (**b**). (**a**, ×45,360; **b**, ×31,500). (With a photographic reduction of 18%).

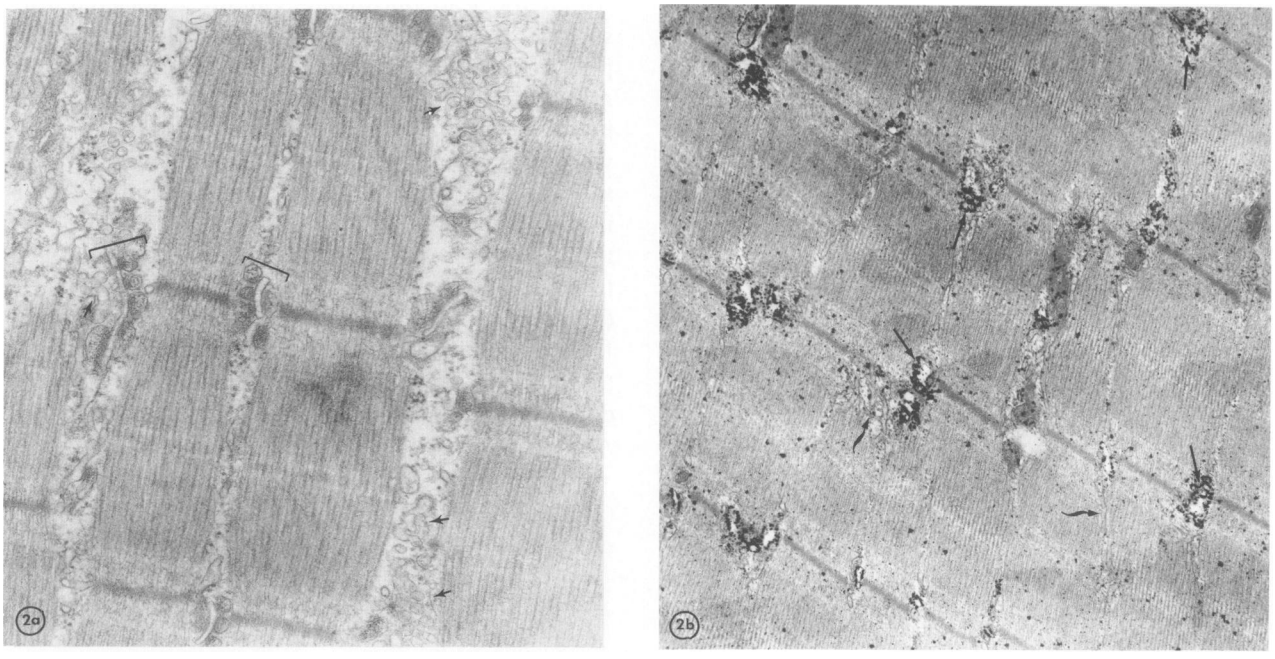

Figure 2—Dystrophic chicken pectoral muscle: T system and derivatives. **a**—shows a control section not incubated for demonstration of ATPase activities. The pathologic findings in this muscle consist of 1) longitudinal rather than transverse orientation of the T system at the triads (*brackets*), 2) a tubular network with extensive vesicular budding or invaginations (*arrows*), 3) triad structures flanked by two or more sarcoplasmic reticulum cisternae, which exhibit typical junctional sarcoplasmic reticulum morphologic appearance, ie, a fine reticular electron-dense pattern. The latter is moderately frequent in this disease. **b**—demonstrates the staining pattern after slices of muscle were incubated with the same medium as in Figure 1. Electron-dense staining precipitate is associated with transverse components of the T system as well as its proliferated derivatives. Dilated microcystic stained structures with scalloped and tortuous perimeters are seen (*arrow*). Precipitates are not associated with sarcoplasmic reticulum (*curved arrow*) in this condition. Minimal background staining is present over mitochondria and myofilaments. (**a**, ×37,260; **b**, ×28,980). (With a photographic reduction of 37%)

myofilaments. The absence of staining in control experiments, where the "basic" ATPase was inactivated by heat, or where unsuccessful attempts were undertaken to preferentially bind lead to surface membrane by exposure of the tissue slices first to lead in the absence of ATP and then to phosphate solution, were compatible with the suggestion that lead phosphate deposit formation was enzyme mediated. In agreement with our previous findings in the normal chicken,[6] a different staining pattern was obtained when Ca^{2+}-ATPase activity associated with sarcoplasmic reticulum was assayed in unfixed tissue in the presence of the Ca^{2+}-ionophore A23187. Under these conditions, the staining pattern was similar to that observed in normal chicken in that electron-dense deposits occurred in the lumen of lateral terminal cisternae of the triads and the longitudinal network of sarcoplasmic reticulum (Figure 3).

Enzymatic Activities of "Crude" Microsomal Fractions

The yield of crude "microsomal" fractions obtained by differential pelleting of the muscle homogenates of 7- and 90-day-old chicken corresponded to about 0.9 and 1.2 mg protein/g muscle for normal and dystrophic chicken, respectively (Table 1). Microsomal fractions were analyzed for Mg^{2+}- or Ca^{2+}-activated ("basic") ATPase activity, an enzyme found to be associated with plasmalemma and T-system membranes of normal[6] and dystrophic chicken (Figures 1 and 2). Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase) activity in the presence of the ionophore A23187, and ^{32}P-labeled phosphoenzyme intermediate levels in the presence of Ca^{2+}, were measured to estimate the sarcoplasmic reticulum Ca^{2+}-ATPase content of the microsomal fractions (Figure 3).[6,20] Ca^{2+}-ATPase activity and ^{32}P-phosphoenzyme levels were lower in 7- than in 90-day-old chicken in both the dystrophic and control lines (Table 1). In contrast, the specific activity of "basic" ATPase was considerably higher in the immature birds. The decrease in specific activity of "basic" ATPase, together with the concomitant increase in Ca^{2+}-ATPase activity and ^{32}P-phosphoenzyme levels, indicated that in the microsomal fractions isolated from 90-day-old chicken the sarcoplasmic reticulum membrane content had increased at the cost of that of the surface membranes.

In addition, Table 1 shows the specific activities of

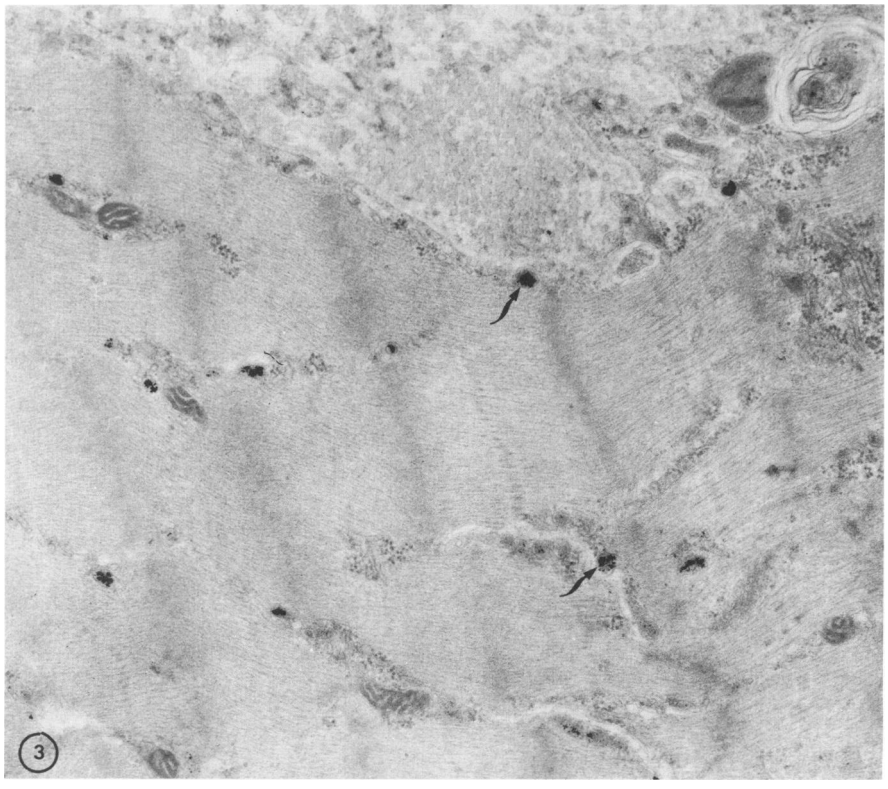

Figure 3—Dystrophic chicken pectoral muscle: Sarcoplasmic reticulum. Fresh muscle slices were incubated in buffer A to which was added the ionophore A23187 (1 μ/ml), 3 mM Tirone, 2 mM Pb^{2+}, 5 mM Mg^{2+}, 0.05 mM Ca^{2+}, and 3 mM ATP. The lead phosphate staining deposits are concentrated within the sarcoplasmic reticulum lumen of the triads and peripheral couplings with the plasmalemma (*curved arrow*). (×29,290) (With a photographic reduction of 15%)

5′-nucleotidase, leucyl β-naphthylamidase, adenylate cyclase, and guanylate cyclase, four enzymes generally believed to be associated with cell membranes of tissues including muscle. Guanylate cyclase[21,22] and leucyl β-naphthylamidase[23,24] have been reported to be present in soluble and membrane-bound forms. Cytosol and microsomal fractions, as well as total muscle homogenates, were therefore assayed for the presence of these two activities. Adenylate cyclase and 5′-nucleotidase, two membrane-bound enzymes, were measured in microsomal fractions and total muscle homogenates of the dystrophic and normal lines.

Results obtained with these four enzymatic activities may be summarized as follows. First, the four enzymes displayed similar specific activities when the homogenates and microsomal fractions of 7-day-old dystrophic and control lines were analyzed. Second, in homogenates of 90-day-old dystrophic chicken, specific activities of adenylate cyclase, guanylate cyclase, and leucyl β-naphthylamidase increased, whereas decreases in specific activity were measured in homogenates of mature normal muscle. Third, a different developmental pattern in the two lines was also indicated when enzymatic activities in microsomal fractions isolated from pectoral muscle in 90-day-old dystrophic and normal lines were compared. In the control line 412, specific activities of all four enzymes decreased in microsomal fractions with maturation of the birds by a factor of two or more. In contrast, a more complex developmental pattern was observed in the dystrophic line. Whereas specific activity of 5′-nucleotidase, like that of the "basic" ATPase of the dystrophic microsomal fraction, decreased, paralleling the maturation pattern seen in the normal line, the specific activity of leucyl β-naphthylamidase remained high in the mature dystrophic microsomal fraction. Adenylate cyclase activity decreased in the dystrophic fractions with age but remained, nevertheless, at a higher level than that observed in the normal animals. Guanylate cyclase showed a marked increase in activity with age in the dystrophic microsomal fractions rather than a decrease as seen in the microsomal fractions from normal birds. Fourth, quantitative differences were observed in the developmental pattern between the soluble and membrane-bound forms of leucyl β-naphthylamidase and guanylate cyclase. Whereas

Table 1—Enzymatic Properties of Microsomes Isolated from Pectoral Muscle of 7- and 90-Day-Old Normal and Dystrophic Chicken*

	Fraction	7 days		90 days	
		Normal	Dystrophic	Normal	Dystrophic
Yield (mg protein/g muscle)	Microsomes	0.9	1.2	0.95	1.25
Ca^{2+}-ATPase (μmol/mg protein/min)	Microsomes	1.0 ± 0.3	0.5 ± 0.3	2.6 ± 0.4	2.9 ± 0.6
^{32}P-phosphoenzyme (nmol ^{32}P/mg protein)	Microsomes	1.4 ± 0.2	1.0 ± 0.3	2.3 ± 0.7	2.6 ± 0.3
"Basic" ATPase (μmol/mg protein/min)	Microsomes	5.0 ± 1.0	7.0 ± 1.0	1.1 ± 0.5	0.4 ± 0.15
5′-nucleotidase					
(μmol/g muscle/min)	Homogenate	0.03	0.02	0.01	0.02
(μmol/mg protein/min)	Microsomes	0.03	0.03	0.015	0.02
Adenylate cyclase					
(nmol/g muscle/min)	Homogenate	2.5 ± 0.5	2.7 ± 0.6	1.7 ± 0.5	3.7 ± 0.9†
(pmol/mg protein/min)	Microsomes	210 ± 45	270 ± 60	45 ± 15	100 ± 25†
Guanylate cyclase					
(nmol/g muscle/min)	Homogenate	0.8 ± 0.1	0.65 ± 0.1	0.2 ± 0.1	0.9 ± 0.15†
(nmol/g muscle/min)	Cytosol	0.4 ± 0.1	0.4 ± 0.1	0.08 ± 0.02	0.24 ± 0.05†
(pmol/mg protein/min)	Microsomes	6.2 ± 1.0	5.0 ± 0.9	3.0 ± 0.7	12.0 ± 3†
Leucyl β-naphthylamidase					
(μmol/g muscle/min)	Homogenate	0.48 ± 0.05	0.6 ± 0.05	0.37 ± 0.03	0.8 ± 0.05†
(μmol/g muscle/min)	Cytosol	0.36 ± 0.04	0.46 ± 0.05	0.35 ± 0.02	0.6 ± 0.07†
(nmol/mg protein/min)	Microsomes	33 ± 10	30 ± 10	8 ± 2	28 ± 6†

* Crude microsomal fractions were prepared from pooled (7 day) or individual (90 day) pectoral muscle, and enzymatic assays were carried out as described in Methods and Materials. The data are the average of three and six determinations ± SE, obtained with five pooled 7-day-old normal and dystrophic chickens each and two 90-day-old normal and dystrophic chicken each, respectively.

† $P < 0.05$ (dystrophic versus normal value, as determined by the Student t test).

in the microsomal fractions leucyl β-naphthylamidase activity decreased fourfold in normal animals but remained high with age in dystrophic animals, only small developmental changes in specific amidase activity were observed in the cytosol fractions of the two lines. Soluble and particulate guanylate cyclase activity decreased in normal chicken. In the dystrophic line, the specific activity of guanylate also decreased with age in the cytosol fraction. In contrast, the microsomal specific activity of this enzyme increased two- to threefold in dystrophic muscle.

Enzymatic Properties of Sucrose Gradient Fractions

Crude microsomal fractions obtained from 90-day-old normal and dystrophic chicken were placed on sucrose gradients and separated into four fractions of different buoyant density. In dystrophic chicken, about 25% of the microsomal protein was recovered from the 0.3 M/25% (Fraction 1) and 25%/30% (Fraction 2) sucrose interfaces, as compared to less than 10% in normal chicken (Figure 4). Gradient fractions were analyzed for Mg^{2+}- or Ca^{2+}-activated ("basic") ATPase activity, Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase) activity, and for their ability to form a ^{32}P-labeled phosphoenzyme intermediate in the presence of Ca^{2+}. In agreement with previous studies,[6,25-28] "basic" ATPase activities of Fractions 1 and 2 were appreciably higher than those of Fractions 3 and 4. Conversely, ^{32}P-phosphoenzyme levels and specific activities of the Ca^{2+}-ATPase activity of Fractions 2–4 were higher than those of Fraction 1.

Figure 4 also shows the sedimentation pattern of the four other surface-membrane-associated enzymes investigated in crude microsomal fractions of 7- and 90-day-old normal and dystrophic chicken (Table 1). Of these, 5′-nucleotidase activity showed a distribution identical to that of "basic" ATPase. The sedimentation pattern of leucyl β-naphthylamidase was qualitatively similar to the patterns of "basic" ATPase and 5′-nucleotidase in that its specific activity was highest in Fractions 1 and 2. However, significant quantitative differences were noticed when normal and dystrophic chicken were compared. Leucyl β-naphthylamidase activity was greatly elevated in the four sucrose fractions of dystrophic chicken. The sedimentation patterns of guanylate cyclase and adenylate cyclase activities differed in an additional aspect. Besides increased levels in dystrophic chicken, these two enzymes displayed nearly identical specific activities in the four sucrose gradient fractions. Enzymatic analysis of the four sucrose fractions suggested, therefore, that there were significant differences in the relative levels and subcellular distributions of enzymatic activities thought to be associated with the surface membranes of 90-day-old normal and dystrophic chicken.

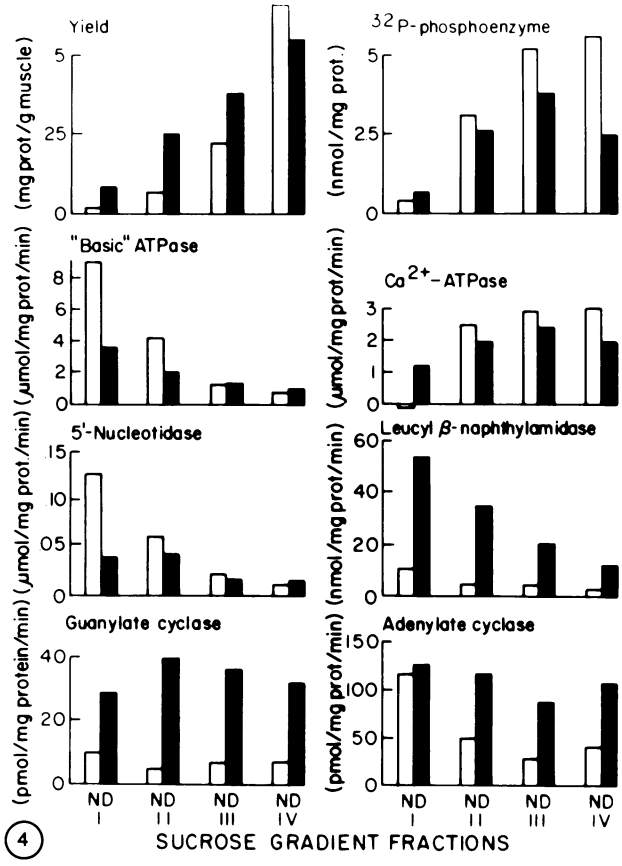

Figure 4—Enzymatic properties of sucrose gradient fractions. Membranous fractions were isolated from pectoral muscle of 90-day-old normal (*N*) and dystrophic (*D*) chicken and assayed for protein and enzymatic activity as described in Materials and Methods. Two representative preparations are shown.

Discussion

A characteristic clinical property of dystrophic chickens is their inability to rise from the supine position. Using an "exhaustion score" test, Wilson et al[29] found that dystrophic line 413 and normal line 412 birds can be statistically distinguished by 10 days after hatching. Virtually none of the dystrophic chickens could right themselves by 30 days. The dystrophic pectoral muscle in these animals showed proliferation of its surface components, the plasmalemma and T system.[3] Cytochemical studies of this paper suggested that the activity of Mg^{2+}- or Ca^{2+}-activated "basic" ATPase was associated with the proliferated surface membrane structures of 90-day-old dystrophic muscle. In addition, the present study showed that there existed significant differences in specific activities of several surface-membrane-associated enzymes in homogenates and microsomal fractions isolated from pectoral muscle of 90-day-old normal and dystrophic chicken. These differences were not observed in fractions of 7-day-old normal and dystrophic chicken.

Enzymatic analysis of the microsomal fractions derived from normal and dystrophic pectoral muscle showed a complex behavior. The surface membrane content in both normal and dystrophic microsomes appeared to decrease with maturation as indicated by the increase in the levels of Ca^{2+}-ATPase and ^{32}P-phosphoenzyme activities (Table 1). An altered sarcoplasmic reticulum Ca^{2+} transport system appeared to be unlikely, since Ca^{2+} uptake by dystrophic microsomes was normal (Malouf and Meissner, unpublished studies), in agreement with previous studies.[30,31] In sucrose gradients, specific activities of "basic" ATPase, 5'-nucleotidase, and leucyl β-naphthylamidase declined with increasing buoyant density, which suggested that the surface membrane contents of sucrose gradient Fractions 3 and 4 were lower than those of Fractions 1 and 2. In support of this interpretation, it was found that Fractions 3 and 4 were enriched in sarcoplasmic reticulum, as indicated by the increased levels of ^{32}P-phosphoenzyme and Ca^{2+}-ATPase activity. On the other hand, the existence of nearly equal surface membrane contents of the four sucrose fractions was suggested when guanylate and adenylate cyclase activities were compared. One explanation for this apparent discrepancy would be a different subcellular distribution of surface-membrane-associated enzymes, as observed in other tissues such as liver.[32] In this regard, it is of interest that the surface membrane of skeletal muscle is thought to be composed of several distinct elements with differing sedimentation properties[23] and enzyme contents.[33]

The present study suggests that several surface-membrane-associated enzymes followed different maturation patterns in normal and dystrophic chicken. The specific activities of "basic" ATPase and 5'-nucleotidase in dystrophic microsomal fractions exhibited developmental patterns similar to those seen in normal animals of corresponding age. This pattern reflected a high specific activity of the two enzymes in the newly hatched animals and a lower activity in the more mature animals. Leucyl β-naphthylamidase, guanylate cyclase, and adenylate cyclase, on the other hand, exhibited specific activity patterns in the dystrophic microsomal fraction that might have reflected a persistent undifferentiated embryonic state. In all three cases, similar, relatively high levels in specific activity were observed in 7-day-old normal and dystrophic lines. In contrast, significant differences were noted in microsomal fractions of 90-day-old animals. In the normal control line, microsomal specific activities decreased two- to four-

fold, whereas in dystrophic line activities decreased less severely (adenylate cyclase), remained at about the same level (leucyl β-naphthylamidase), or actually increased (guanylate cyclase).

Differences in adenylate cyclase activity between dystrophic and control tissues have been previously observed. Horvarth and Levin[34] found that the muscle homogenates of both normal and dystrophic 18-day-old embryos contained high levels of adenylate cyclase activity. In normal muscle, activity decreased rapidly with development, reaching a low level at about 100 days, as observed in the present study. In dystrophic muscle homogenates, activity declined to a lesser degree, increased again by 100 days, and remained high in the mature bird. A different developmental pattern was observed by Rodan et al.[35] In partially purified surface membrane fractions isolated from 16-day embryos and 8- and 42-day-old dystrophic chicken, adenylate cyclase increased progressively to reach at 42 days an activity twice or more that of the control. One reason for this different developmental pattern may have been that Rodan et al used lines that differ from those of the present study and the study of Horvarth and Levin.[34] In a more recent study, Rodan et al[37] found that the ratio of specific activities of adenylate cyclase of microsomal fractions derived from dystrophic and normal muscle increased steadily from 0.69 at 1 week after hatching to 9.4 at 9 weeks. To our knowledge, the activity of guanylate cyclase has not been previously studied in dystrophic chicken muscle. It is difficult to speculate over the reasons behind its increase as found in this study, since the biologic role of guanylate cyclase and its regulation in normal muscle are not well defined.

Various enzyme systems have been reported altered in avian lines inflicted with dystrophy (reviewed by Wilson et al[29] and Cosmos et al[37]). Wilson et al[29] have proposed that increases in the activity of lysosomal cathepsins,[38] followed by muscle protein degradation in dystrophic humans and animals, are secondary to the dystrophic state. Failure of pectoralis muscle in dystrophic chicken to undergo differentiation from an embryonic state to an adult homogeneous fast-twitch muscle type has been hypothesized as another explanation for some of these alterations. About 4–12 weeks *ex ovo*, the normal pectoralis muscle acquires biochemical and histochemical characteristics of fast-twitch fibers, with high levels of glycolytic enzyme activity. The dystrophic muscle, like embryonic muscle, maintains high levels of oxidative enzyme activity, while glycolytic enzymes such as the phosphorylase and aldolase remain less active.[29,37,39] The persistent embryonic pattern seen in the membrane-bound enzyme activities of leucyl β-naphthylamidase, guanylate, and adenylate cyclases in isolated dystrophic fractions in this study may be, therefore, another reflection of such a lack of differentiation. On the other hand, a "normal" developmental pattern was displayed by "basic" ATPase and 5′-nucleotidase activities, which suggests that additional factors will have to be considered to describe the pathologic state of dystrophic chicken.

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